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Nagarajan, Shunmugam ; Vohra, Twinkle ; Loffing, Johannes ; Faresse, Nourdine

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# **Protein Phosphatase 1 $\alpha$ Enhances Renal Aldosterone signaling via Mineralocorticoid Receptor Stabilization**

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## **Abstract**

Stimulation of the mineralocorticoid receptor (MR) by aldosterone controls several physiological parameters including blood pressure, inflammation or metabolism. We previously showed that MR turnover constitutes a crucial regulatory step in the responses of renal epithelial cells to aldosterone. Here, we identified Protein Phosphatase 1 alpha (PP1 $\alpha$ ), as a novel cytoplasmic binding partner of MR that promotes the receptor activity. The RT-PCR expression mapping of PP1 $\alpha$  reveals a high expression in the kidney, particularly in the distal part of the nephron. At the molecular level, we demonstrate that PP1 $\alpha$  inhibits the ubiquitin ligase Mdm2 by dephosphorylation, preventing its interaction with MR. This results in the accumulation of the receptor due to reduction of its proteasomal degradation and consequently a greater aldosterone-induced Na<sup>+</sup> uptake by renal cells. Thus, our findings describe an original mechanism involving a phosphatase in the regulation of aldosterone signaling and provide new and important insights into the molecular mechanism underlying the MR turnover.

## Introduction

The mineralocorticoid hormone aldosterone is considered as a principal endocrine factor involved in the regulation of ions homeostasis and blood pressure. Aldosterone mediates its effects through the Mineralocorticoid Receptor (MR), a 984 amino acids protein belonging to the steroid/thyroid hormone receptor superfamily and deriving from the *Nr3C2* gene (Arriza, Weinberger, Cerelli et al., 1987, Rogerson, Dimopoulos, Sluka et al., 1999). The MR protein is divided in three domains; the N-terminal domain (NTD) involved in the control of the transcriptional activity of the receptor; the central DNA-binding domain (DBD) involved in the binding of the specific response element found on the promoter of MR target genes; and finally a ligand-binding domain (LBD) responsible for the selectivity of hormone binding (Faresse, 2014). Upon hormonal activation, MR is translocated into the nucleus either as a homodimer, or associated to the Glucocorticoid Receptor (GR) as a heterodimer (Ou, Storrang, Kushwaha et al., 2001). In the nucleus, MR recruits co-factors to induce the transactivation and regulation of hundreds of target genes (Gekle, Bretschneider, Meinel et al., 2014, Verrey, Pearce, Pfeiffer et al., 2000). These genes present a palindromic DNA sequence common for GR and MR called glucocorticoid response element (GRE) within their promoter (Fuller, Lim-Tio and Brennan, 2000). The ligand activation mediates also a proteasomal degradation of MR allowing a rapid turnover of the activated receptor and attenuates the transcriptional response to aldosterone (Faresse, Ruffieux-Daidie, Salamin et al., 2010, Faresse, Vitagliano and Staub, 2012, Yokota, Shibata, Kobayashi et al., 2004).

Among the different tissues expressing MR, the principal cells of the aldosterone sensitive distal nephron (ASDN) are the best characterized targets of aldosterone. These cells build a polarized epithelium allowing a vectorial transport of ions and water between the urinary apical and the interstitial basolateral sides. In these cells, activation of MR by aldosterone enhances the electrogenic  $\text{Na}^+$  and water reabsorption and luminal  $\text{K}^+$  secretion. These characteristics are endorsed mainly by the apical expression of the epithelial sodium channel (ENaC), the renal outer medullary potassium channel (ROMK), and the basolateral expression of the Na-K-ATPase (Meneton, Loffing and Warnock, 2004). The importance of the MR in ion homeostasis and blood pressure control has been demonstrated by either loss-of-function mutations in the *Nr3C2* gene, causing autosomal dominant pseudohypoaldosteronism type 1 (adPHA1) (Geller, Rodriguez-Soriano, Vallo Boado et al., 1998) or gain-of-function mutations leading to a severe inherited

form of early-onset hypertension, which is exacerbated during pregnancy (Geller, Farhi, Pinkerton et al., 2000). Other pathologies as renal and cardiac injuries (Luther, Luo, Wang et al., 2012), cerebral aneurysm formation (Tada, Yagi, Kitazato et al., 2010), chorioretinopathy (Zhao, Celerier, Bousquet et al., 2012), and epidermis abnormalities (Farman, Maubec, Poeggeler et al., 2010) have been shown to be associated also with an over-activation of MR signaling. In this context, numerous clinical studies demonstrated the efficiency of MR antagonists in the prevention of disease progression (Pitt, Zannad, Remme et al., 1999, Bolignano, Palmer, Navaneethan et al., 2014, Weinberger, Roniker, Krause et al., 2002). Therefore, the maintenance of the body homeostasis is closely related to a tight regulation of MR signaling that occurs at the level of ligand availability, the co-factors, the MR nucleo-cytoplasmic shuttling, the cross-talk with additional signaling pathways and finally the post-translational modifications (PTMs) of the receptor. We previously demonstrated in renal epithelial cells that aldosterone stimulates a rapid phosphorylation of MR via ERK1/2 that is critical for MR ubiquitylation state and turnover (Faresse et al., 2012). In addition, it was described that the specific S843 phosphorylation of MR, occurring exclusively in intercalated cells of the nephron, corresponds to a switch of the aldosterone response from K<sup>+</sup> secretion to Na<sup>+</sup> reabsorption by the kidney (Shibata, Rinehart, Zhang et al., 2013). However, the molecular mechanisms characterization of this modification is still puzzling and was suggested to involve the kinase WNK4 and the protein phosphatase 1 (PP1) (Shibata et al., 2013).

The PP1 holoenzyme consists of a catalytic subunit (PP1c) and one or more co-factors. PP1c has four isoforms: PP1 $\alpha$  (encoded by *ppp1CA*), PP1 $\beta$  (encoded by *ppp1CB*) and PP1 $\gamma$ 1 and  $\gamma$ 2 (splice variants of *ppp1CC*). These isoforms share a high degree of homology and their specificity is dependent on their co-factors, although the isoforms themselves may also drive these interactions and influence the substrate selection (Ceulemans and Bollen, 2004, Chiang, Heck, Dobrev et al., 2016). Expression and/or activity of several steroid receptors, such as the androgen receptor, the pregnane X receptor or the estrogen receptor-  $\alpha$  have been shown to be regulated by PP1 $\alpha$  (Liu, Han, Gulla et al., 2016, Sugatani, Hattori, Noguchi et al., 2014, Bollig, Xu, Thakur et al., 2007). In this study, we identified PP1 $\alpha$  as a novel MR binding protein that stabilizes the expression and the activity of the receptor. We investigated the basis of this mechanism and found that the ubiquitin ligase Mdm2, responsible for the MR ubiquitylation and degradation, is dephosphorylated and inactivated by PP1 $\alpha$ . Thus, PP1 $\alpha$  defines a new component

of the aldosterone signaling that enhances aldosterone stimulation through controlling MR expression levels and consequently the Na<sup>+</sup> uptake by renal epithelial cells.

## Results

### *PP1 $\alpha$ binds to cytoplasmic MR in renal epithelial cells*

To assess the organ distribution of PP1 $\alpha$ , a qRT-PCR analysis was performed to evaluate PP1 $\alpha$  mRNA expression levels in different tissues of normal mice (Figure 1A). We found that PP1 $\alpha$  expression was high in lung, kidney and white adipose tissue, and very low or undetectable in spleen, muscle and pancreas (Figure 1A). To estimate the expression of PP1 $\alpha$  in renal epithelial cells, we isolated mRNA from freehand microdissected tubules from mouse kidneys. We found that PP1 $\alpha$  is highly expressed in the distal part of the nephron, including the distal convoluted tubule (DCT) and the collecting system (CS), tubules segments showing also the highest expression of MR and designated as the aldosterone-sensitive distal nephron (Figure 1B). Based on previous studies showing that PP1 $\alpha$  interacts and regulates certain steroid receptors (Liu et al., 2016, Sugatani et al., 2014, Bollig et al., 2007), we hypothesized that PP1 $\alpha$  may interact also with MR in renal epithelial cells. To verify this hypothesis, we cotransfected S-tagged MR and analyzed its association with Flag-tagged PP1 $\alpha$  in HEK-293 cells. As shown in Figure 2A, MR co-immunoprecipitated with PP1 $\alpha$ , and this interaction was decreased by aldosterone treatment. Due to the lack of suitable antibody for immunoprecipitation of non-tagged proteins, we confirmed the endogenous interaction between MR and PP1 $\alpha$  by proximity ligation assay (PLA) in mouse cortical collecting duct cells (mCCD<sub>cl1</sub>). To validate this assay, we first showed that co-transfection of MR and PP1 $\alpha$  in HEK-293 cells resulted in a prominent PLA signal in the cytoplasm (Figure 2B bottom); and omission of transfected PP1 $\alpha$  plasmids resulted in the absence of signal (Figure 2B top). This association was also confirmed endogenously in mCCD<sub>cl1</sub> cells, since MR and PP1 $\alpha$  provided a PLA signal in the cytoplasm that was reduced by aldosterone stimulation (Figure 2C). Quantitative analysis demonstrated that PLA signal was significantly reduced (about 50%) when mCCD<sub>cl1</sub> cells were treated with aldosterone (Figure 2D).

### *PP1 $\alpha$ activity prevents MR proteasomal degradation*

As PP1 $\alpha$  has been shown to regulate the expression of certain steroid receptors, we tested whether this phosphatase controls also MR stability. To answer this question, we used tautomycin, a potent and specific pharmacological inhibitor of PP1 (Swingle, Ni and Honkanen, 2007). Treatment of mCCD<sub>cl1</sub> cells with tautomycin induced a dramatic decrease in endogenous

MR expression in a dose dependent manner, without affecting the expression of PP1 $\alpha$  (Figure 3A). We also demonstrated that this effect is time dependent (Figure 3B). To ascertain the specific effect of PP1 $\alpha$ , we showed that overexpression of increasing amounts of PP1 $\alpha$  produced a dose-dependent increase in the expression level of MR (Figure 3C). Furthermore, the co-transfection of I1, an endogenous inhibitor of PP1, completely abolished the PP1 $\alpha$ -induced stabilization of MR, and reduced the level of the receptor (Figure 3D). The tautomycin-dependent decrease of steroid receptors expression has been reported to involve the ubiquitin/proteasome system (Liu et al., 2016). We confirmed these observations by showing that PP1 $\alpha$  inhibition increased the polyubiquitylation of MR (Figure 3E) and inhibition of the proteasome by MG132 prevented the tautomycin-induced degradation of endogenous MR (Figure 3F). These data suggested that PP1 $\alpha$  binding to the receptor may prevent the induction of MR polyubiquitylation and degradation. It has been described that PP1 $\alpha$  binds to its substrates via the consensus docking motif partners, R/K-x(0,1)-V-x-F (Garcia, Cayla, Caudron et al., 2004). The DNA-binding domain of MR presents a conserved potential PP1 $\alpha$  consensus binding motif KVFF (Figure 4A). To investigate the potential role of this motif, we mutated the essential residues V625 and F627 to alanine (KVFF to KAFA). We found that the mutation has no effect on MR nuclear translocation after aldosterone stimulation (Figure 4B). We also found that mutated MR preserved its ability to interact with the receptor and that aldosterone reduced this association (Figure 4C). Finally, the MR mutant was stabilized by PP1 $\alpha$  in a similar manner to the wild-type form (Figure 4D). Taken together, these data suggested that PP1 $\alpha$  binds to MR and stabilizes its expression independently of the substrate recognition sequence KVFF.

#### *PP1 $\alpha$ stabilizes MR expression by reducing Mdm2 activation*

To investigate the molecular mechanisms explaining the MR stabilization by PP1 $\alpha$ , we focused on the ubiquitin ligase Mdm2. This enzyme has been shown to induce the degradation of several steroid receptors (Liu et al., 2016, Sengupta and Wasylyk, 2001, Duong, Boulle, Daujat et al., 2007, Heyne, Heil, Bette et al., 2015) and its activity is regulated by PP1 $\alpha$  (Liu et al., 2016, Lee, Lim, Min et al., 2007). To confirm the role of PP1 $\alpha$  on Mdm2 activity, we evaluated the activating phosphorylation occurring on the residue S166. We found that inhibition of PP1 $\alpha$  by tautomycin led to an increase of endogenous Mdm2 phosphorylation in renal epithelial cells (Figure 5A). Conversely, overexpression of PP1 $\alpha$  induced a decrease of S166 phosphorylation levels (Figure 5B). Then, we tested whether Mdm2 interacts with MR and if PP1 $\alpha$  activity



regulates this association. Using the PLA, we found that endogenous Mdm2/MR association was small in control conditions, while treatment with tautomycin increased significantly this interaction (Figure 5C and D). This observation suggested that PP1 $\alpha$  activity prevents the recruitment of Mdm2 to MR. To test whether MR is a substrate for Mdm2, we first demonstrated by immunoprecipitation experiments that Mdm2 increased the polyubiquitylation of MR (Figure 5E). This modifications reduced MR expression, since co-transfection of MR with an increasing dose of Mdm2 reduced MR expression level in a dose dependent manner (Figure 5F). However, over-expression of PP1 $\alpha$  abrogated completely the effects of Mdm2 and restored the MR expression (Figure 5G). Similarly, inhibition of Mdm2 activity by Nutlin-3, abolished the tautomycin induced degradation of MR (Figure 5H). All together, we demonstrated that PP1 $\alpha$  activity prevents the binding of Mdm2 to MR and consequently the polyubiquitylation and degradation of the receptor.

#### *The cellular responses to aldosterone are enhanced by PP1 $\alpha$*

Given that PP1 $\alpha$  stabilizes the expression level of MR, we questioned whether PP1 $\alpha$  affects also its activity. We first showed that stabilization of MR by PP1 $\alpha$  increased the presence of MR in the nucleus, both at basal and after aldosterone stimulation (Figure 6A). Conversely, treatment with tautomycin abrogated totally the aldosterone nuclear accumulation of MR (Figure 6A). The higher accumulation of MR in the nucleus suggested that PP1 $\alpha$  affects the transcription of target genes. To verify this hypothesis, we examined whether PP1 $\alpha$  could influence MR transcription by luciferase assay using the aldosterone-responsive reporter gene mouse mammary tumor virus (MMTV)-luc. Consistent with the nuclear accumulation of MR, PP1 $\alpha$  overexpression resulted in a significant increase of the basal as well as the aldosterone-dependent transcription (Figure 6B). To test the effect of PP1 $\alpha$  on the intrinsic transcriptional activity of MR and its ligand affinity, we performed a luciferase assay using several doses of aldosterone. We found that PP1 $\alpha$  overexpression affected slightly but significantly the affinity of MR to aldosterone (IC<sub>50</sub>: MR= 0.19nM  $\pm$ 0.05; MR+PP1 $\alpha$ = 0.08nM  $\pm$ 0.02,  $p$  value=0.02) (Figure 6C). As these PP1 $\alpha$  effects were assumed to be related to Mdm2 inhibition, we performed a similar experiment by co-transfecting Mdm2. We found that Mdm2 reduced significantly the transcriptional activity of MR, and this inhibition was relieved by co-transfection of PP1 $\alpha$  (Figure 6D). To investigate the endogenous significance of PP1 $\alpha$  in renal epithelial cells, we followed the synthesis of the aldosterone-induced gene Sgk1 in mCCD<sub>cl1</sub> cells (Bhargava, Fullerton, Myles et al., 2001). As

anticipated, Sgk1 mRNA and protein levels were significantly upregulated upon aldosterone stimulation and this induction was drastically inhibited by co-treatment with tautomycin (Figure 6E).

As the aldosterone-mediated transcription was affected by PP1 $\alpha$ , we tested the impact of PP1 $\alpha$  inhibitor on the aldosterone-stimulated Na<sup>+</sup> reabsorption via the epithelial Na<sup>+</sup> channel (ENaC). In this context, we determined the transepithelial current of polarized mCCD<sub>cll</sub> grown of collagen-coated semipermeable filters (Faresse et al., 2010, Gaeggeler, Gonzalez-Rodriguez, Jaeger et al., 2005). We found that the increase of Na<sup>+</sup> current mediated by aldosterone was significantly reduced when cells were co-treated with tautomycin (Figure 7A). The fact that the transepithelial resistance (Figure 7B) was not significantly affected by tautomycin treatment suggested that the drug did not perturb the epithelial tight junctions. Therefore, the observed variations in Na<sup>+</sup> flux were likely attributable to variations in the transepithelial transport of Na<sup>+</sup>. Hence, these results suggest that PP1 $\alpha$  enhances the cellular response to aldosterone and consequently increase the Na<sup>+</sup> transport by ENaC in renal epithelial cells.

## Discussion

Our study proposes PP1 $\alpha$  as a novel protein partner of the non-activated cytoplasmic MR. This phosphatase stabilizes MR expression and renders the renal epithelial cells more responsive to aldosterone stimulation. Interestingly, we found that PP1 $\alpha$  is highly expressed in the kidney, suggesting a regulatory role of this phosphatase in Na<sup>+</sup> handling and consequently in blood pressure control. Inhibiting PP1 $\alpha$  activity with the pharmacological inhibitor Tautomycin or by overexpression of the specific inhibitor I1 reduced both the transfected and the endogenous MR expression. At the molecular level, we showed that PP1 $\alpha$  inactivates Mdm2 via dephosphorylation of the serine 166, reducing the Mdm2-triggered poly-ubiquitylation and proteasomal degradation of MR.

We previously reported that aldosterone activation induces a rapid phosphorylation of MR on several serine residues in the NTD (Faresse et al., 2012). These ERK1/2 mediated phosphorylations are critical for the aldosterone-induced poly-ubiquitylation and degradation of the receptor. However, according to our findings, MR seems not to be a direct substrate of PP1 $\alpha$ . First, a mutant form of MR, where the consensus docking motif found within PP1 $\alpha$  substrates was altered, maintained its ability to interact and to be stabilized by PP1 $\alpha$  (Figure 4). Furthermore, we previously showed that MR phosphorylation is reflected by an increase of the apparent molecular weight of the receptor on immunoblots (Faresse et al., 2010, Faresse et al., 2012). In this study, we found that PP1 $\alpha$  activity did not affect the aldosterone induced upward shift of MR, indicating an absence of a direct effect on the receptor phosphorylation. However, several ERK1/2-independent phosphorylation sites on MR have been reported (reviewed in (Faresse, 2014)). Thus, we cannot exclude a potential direct effect of PP1 $\alpha$  on ERK1/2-independent sites, such as the serine 843 suggested by a previous report using the purified proteins MR and PP1 (Shibata et al., 2013).

The ubiquitin ligase Mdm2 has been shown to control the expression of several steroid receptors, such as the glucocorticoid receptor (Sengupta and Wasylyk, 2001), the estrogen receptor (Duong et al., 2007), the androgen receptor (Liu et al., 2016), or the vitamin D receptor (Heyne et al., 2015). Here we demonstrated that Mdm2 was also able to increase the poly-ubiquitylation and the degradation of MR, confirming previous reports indicating that Mdm2 may play a role in aldosterone signaling. For instance, it was demonstrated that the aldosterone early target gene Sgk1 regulates the phosphorylation level of Mdm2 at serine 166 (Amato, D'Antona,

Porciatti et al., 2009). Furthermore, in vascular smooth muscle cells, it has been shown that Mdm2 is a mineralocorticoid-responsive gene involved in aldosterone-induced human vascular structural remodeling (Nakamura, Suzuki, Suzuki et al., 2006). However, questions persist about the precise physiological conditions promoting MR degradation by Mdm2, which may contribute to a negative feedback regulation of aldosterone signaling. In this context, we and others have described that ligand stimulation of MR, increases the receptor poly-ubiquitylation and turnover (Faresse et al., 2012, Yokota et al., 2004). However, neither the enzymes nor the target lysine residues on MR were identified. Here we showed that MR stability was sensitive to Mdm2 and given that the kinase Sgk1 regulates Mdm2 activity, this ubiquitin ligase appears to be an interesting candidate requiring further characterization in the context of the MR aldosterone-mediated degradation.

We report in this study that PP1 $\alpha$  is highly expressed in the aldosterone-sensitive distal nephron. Furthermore, by interfering pharmacologically with PP1 $\alpha$  activity, we were able to limit the aldosterone-induced Na<sup>+</sup> uptake by cortical collecting duct cells. These findings shed light on the potential implication of PP1 $\alpha$  in ions homeostasis. The aldosterone-sensitive distal nephron reabsorbs 5%-10% of the filtered Na<sup>+</sup> (Palmer and Schnermann, 2015). This process is highly regulated helping to match urinary Na<sup>+</sup> excretion with dietary Na<sup>+</sup> intake. Inappropriate Na<sup>+</sup> reabsorption by the distal nephron is generally associated with altered blood pressure (Lifton, Gharavi and Geller, 2001). Several kinases have been described to play a critical role in the regulation of signaling pathways and transporters involved in Na<sup>+</sup> reabsorption along the ASDN. However, only few phosphatases have been linked to renal Na<sup>+</sup> handling. It has been shown that pharmacological inhibition of the protein phosphatase 3 (Calcineurin) by tacrolimus activates the renal sodium chloride cotransporter (NCC) and cause hypertension (Hoorn, Walsh, McCormick et al., 2011). Furthermore, genetic ablation of the endogenous PP1 inhibitor I-1 or *ex vivo* incubation of DCTs with the PP1 inhibitor calyculin A drastically affected NCC phosphorylation and blood pressure (Sorensen, Grossmann, Roesinger et al., 2013, Picard, Trompf, Yang et al., 2014). The physiological role of PP1 in blood pressure control is corroborated by observations done in the heart of transgenic mice over-expressing the active PP1-inhibitor I1. The inhibition of PP1 specifically in the heart was associated with enhanced cardiac function and conferred protection against heart failure propensity, similarly to what was described clinically with MR blockers (Pathak, del Monte, Zhao et al., 2005, Zannad, McMurray, Krum et al., 2011).

In summary, our findings proposed PP1 $\alpha$  as a novel regulator of the aldosterone signaling particularly in kidney epithelial cells. This phosphatase renders cells sensitive to aldosterone stimulation by maintaining an appropriate pool of the aldosterone receptor MR. To the best of our knowledge; this is the first report describing the role of PP1 $\alpha$  and its substrate Mdm2 in the control of MR expression level. These findings open new perspectives and raise new questions about the implication of MR turnover in blood pressure control.

## Material & Methods

### *Cell culture*

Mouse cortical collecting duct clone 1 (mCCD<sub>cl1</sub>) cells were cultured in complete medium containing DMEM-F12 (1:1), 5µg/ml insulin, 5µg/ml transferrin, 6×10<sup>-8</sup>M sodium selenate, 5×10<sup>-8</sup> M dexamethasone, 1nM triiodothyronine, 10ng/ml epidermal growth factor, 10mM HEPES, pH 7.4, and 2% FCS. For experiments, cells were seeded and grown in complete growth medium on collagen coated Transwell filters for first 5 days, and then grown for another 5 days in medium containing DMEM-F12 medium (1:1), 5µg/ml insulin, 6×10<sup>-8</sup>M sodium selenate, 3×10<sup>-9</sup>M dexamethasone, 1nM triiodothyronine, and 10mM HEPES, pH 7.4. Before experiments, cells were incubated in minimal medium [DMEM-F12 (1:1)] for overnight and all the treatments were done in minimal medium. (Faresse et al., 2010). HEK293T cells were grown in DMEM supplemented with 10% FCS. The cells were transfected with lipofectamine following the manufacturer`s direction (Invitrogen).

### *In situ proximity ligation assay:*

MR interaction with PP-1  $\alpha$  and MDM2 were detected by in situ proximity ligation assay (PLA) by using commercially available kit (Duolink, Olink Biosciences). HEK293T cells were co-transfected with Stag-MR wt and FLAG-PP1 $\alpha$ . Mouse monoclonal S-tag and Rabbit monoclonal PP1 $\alpha$  antibodies were used to detect MR and PP1 $\alpha$  respectively. In mCCD<sub>cl1</sub>, we used rabbit monoclonal PP1 $\alpha$  antibody and mouse MR antibody to detect endogenous MR/PP1 $\alpha$  interaction in the presence and absence of aldosterone. To detect MR and MDM2 interaction, we used rabbit MDM2 and mouse MR antibodies in mCCD<sub>cl1</sub> treated or not with tautomycin. For the negative controls, either one of the primary antibodies or plasmids were omitted. Number of fluorescent dots were counted per cell visually under fluorescent microscope and expressed as an average from 3 independent staining. For this experiment, the low cell confluency allowed a clear delimitation of each counted cell.

### *Plasmids and antibodies*

Expression vectors for the transfected proteins were pcDNA3.1 Stag-MR wt, and the mutant form of MR (KAFA) was derived from wild-type (wt) MR by site-directed mutagenesis, Myc-Mdm2, FLAG-PP1 $\alpha$  and HA-ubiquitin. The antibodies used were anti-MR (1:100) (Gomez-Sanchez, de Rodriguez, Romero et al., 2006), anti-PP1 $\alpha$  (1:1000 ab52619, Abcam), anti-MDM2 (1:1000, SC-

812, Santa Cruz), anti-P-Mdm2 (1:500, 3521, Cell Signaling), anti-Stag (1:5000, 71549-3, Novagen), anti-FLAG FG4R (1:5000, MA1-91878, Thermo Fisher), anti-Myc 4A6 (1:1000, 05-724, Millipore), anti-HA (1:4000, ab9110, Abcam), anti-SGK1 (1:1000, S5188; Sigma).

#### *Immunoblotting and immunoprecipitation*

Procedures were followed as described previously (Faresse et al., 2012). Briefly, for immunoprecipitation, 500µg proteins were incubated overnight at 4°C under rotation with the respective antibodies and then incubated with protein G-Sepharose beads (Amersham) at 4°C for 1 h. For the pull down assay, S-tagged agarose beads (Novagen) were incubated with the lysate at 4°C for 4 hrs. After five times washing, samples were eluted in 40 µl of 2× Sample Buffer and submitted to 8% SDS-PAGE.

#### *Transcriptional reporter assay*

HEK293T cells were seeded on 24-well tissue culture plates then co-transfected by Lipofectamine with the reporter gene MMTV-luc (kindly provided by P.J Fuller), the constitutive luciferase vector coding for Renilla and the indicated plasmids. After 30h, cells were exposed to aldosterone (10 nM) for 16h. After hormone treatment, cells were washed twice with phosphate-buffered saline (PBS) and lysed with 100 µl of passive lysis buffer (Promega). Luciferase activity was determined by using a luciferase kit (Promega). Luciferase activity was normalized for constitutive Renilla luciferase. The IC50 and its standard deviation was evaluated by the R software using the dose response model LL.4.

#### *Tubules microdissection*

C57BL/6 mice aged between 8-14 weeks were used to map the expression of MR and PP1α along the nephron. The harvesting of the kidneys was performed in accordance to Swiss animal welfare regulations. Mice were anesthetized by isoflurane inhalation and the kidneys were perfused with 10 ml DMEM/ F-12 media (Gibco) followed by 4.5mL liberase (0.06 mg/mL; Roche). The cortical part was cut into thin horizontal sections and digested in 500µl liberase. After washing with ice cold media, segments were manually dissected under stereomicroscope. The tubuli were identified on the bases of their respective morphology. Total RNA was isolated using Absolutely RNA Nanoprep Kit (Agilent) and 20 ng of total RNA was used to generate cDNA (Superscript IV, Thermo Fisher Scientific) using random primers. Each dissected sample was scrutinized for

its purity using specific gene markers for instance Podocalyxin for Glomeruli, Aminopeptidase N (CD 13) for Proximal Tubuli, NKCC2 for Thick Ascending Limb, NCC for distal convoluted tubuli and Aqp-2 for collecting system and 18s RNA was used as the housekeeping gene. Any sample with cross contamination was discarded. The clean samples expressing exclusively their respective nephron marker were finally selected.

#### *Real-time quantitative PCR*

Total RNA was extracted using the RNeasy Mini Kit (Qiagen) and was reverse transcribed using RevertAid™ cDNA Synthesis Kit (Thermo Scientific). Sequences of primers are indicated in Table 1. Quantitative RT-PCR was performed in duplicate for each sample in a Light Cycler 480 Real-Time PCR System by using Master I SYBR Green qPCR Master Mix (Roche), according to the manufacturer's instructions. Relative amounts of mRNA were determined using the Comparative CT Method for quantification and were normalized to GAPDH mRNA expression.

#### *Membrane potential and resistance measurements*

Confluent mCCD<sub>cll</sub> cells were seeded and grown on collagen-coated Transwell filters for the measurement of Na<sup>+</sup> transport in response to aldosterone. Transepithelial potential difference (mV) and transepithelial electrical resistance (Ohm/cm<sup>2</sup>) were recorded under sterile conditions using Millicell-ERS ohmmeter apparatus (Millipore), and the corresponding currents were calculated. In each experiment, there were three filters per condition. Before measuring, cells were treated or not with tautomycin (100nM for 18 hrs), with or without aldosterone (10nM for 4 hrs).

#### *Immunofluorescence analysis*

HEK293T cells were transfected with MR<sup>WT</sup> and MR<sup>KAFA</sup>. After 48 hrs, cells were treated with vehicle or aldosterone (10 nM) for 2 hrs. Afterward, cells were fixed in 4% formaldehyde for 30 min and permeabilized in 0.1% Triton X-100. Then, the cells were incubated with the anti-MR primary antibody overnight at 4°C, followed by the secondary antibody conjugated to FITC for 1h at room temperature. The coverslips were mounted using mounting medium and then were imaged under fluorescence microscope.

#### *Data analysis*



All the experiments were done at least twice, and the representative immunoblots or IF images are shown. Experiments were done in triplicate and all data are presented as mean  $\pm$  S.D and were analyzed by unpaired two-tailed Student's t test. P-values of  $<0.05$  were considered to be significant.

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## Figure legends

**Figure 1:** PP1 $\alpha$  is highly expressed in the kidney. (A) The abundance of PP1 $\alpha$  mRNA expression relative to Gapdh was assessed by qRT-PCR in different organs from 4 mice. (B) The abundance of PP1 $\alpha$  and MR mRNA expression relative to 18s RNA was assessed by qRT-PCR along the nephron. Glomerulus (Glom.), Proximal Tubuli (PT), Descending Limb (DL), Thick Ascending Limb (TAL), Distal Convolved Tubuli (DCT), Collecting System (CS). The values were reported to the maximum of expression (DCT for PP1 $\alpha$  and CS for MR)  $\pm$ SD of triplicates.

**Figure 2:** PP1 $\alpha$  interacts with MR in renal epithelial cells. (A) HEK-293 cells were co-transfected with Stag-MR and Flag-PP1 $\alpha$  and treated with vehicle or aldosterone (10nM) for 3h before lysis. Cell lysates were immunoprecipitated with anti-Flag and blotted with anti-Stag for immunoprecipitation control. Expression level of proteins under investigation was determined by direct immunoblotting (Input). Data shown are representative from 2 independent experiments. (B) PLA assay detecting MR-PP1 $\alpha$  interactions. HEK-293 cells were transfected with MR alone or MR+PP1 $\alpha$  and fixed. After the PLA reaction, coverslips were mounted in the presence of DAPI to counterstain nuclei. (C) PLA assay detecting MR-PP1 $\alpha$  interactions on mCCD<sub>cll</sub> cells treated with vehicle or 10nM aldosterone. (D) Quantitative analysis of PLA signals in mCCD<sub>cll</sub> cells from experience (C). Bars represent the means of discrete dots per cells  $\pm$  SEM (n=80) from 3 independent stainings, \*\*\*P<0.001, control vs. +Aldo.

**Figure 3:** PP1 $\alpha$  prevents MR degradation by the proteasome. (A and B) mCCD<sub>cll</sub> cells were treated with an increasing dose of Tautomycin from 50nM to 200nM for 16h, or at indicated time with 100nM. Cell lysates were analyzed by immunoblotting using antibodies against MR and actin as a loading control. (C) HEK-293 cells were co-transfected with Stag-MR and increasing doses of Flag-PP1 $\alpha$ . The expression of proteins under investigation was determined by direct immunoblotting with anti-Stag or anti-Flag antibodies and actin as a loading control. (D) Stag-MR, Flag-PP1 $\alpha$  were co-transfected with or without Myc-I1 in HEK-293 cells. Cell lysates were analyzed by direct immunoblotting with anti-Stag, anti-Flag and anti-Myc antibodies and actin as a loading control. (E) HEK-293 cells were co-transfected with Stag-MR and HA-Ub and treated with vehicle or Tautomycin (100nM) for 16h and MG132 (10 $\mu$ M the last 6h). Cell lysates were submitted to Pull-Down (PD) with Stag agarose beads and the ubiquitylated forms of MR were

analyzed by immunoblotting with anti-HA antibody. Expression level of MR was determined by direct immunoblotting (Input) and actin as loading control. (F) mCCD<sub>cll</sub> cells were treated with vehicle or Tautomycin (100nM for 16) in presence or absence of the proteasome inhibitor MG132 (10μM for the last 6h). MR expressed was assessed by immunoblot using anti-MR antibody and actin as loading control. (A to F) Immunoblots shown are representative from 2-4 independent experiments.

**Figure 4:** PP1α binds to MR independently to its KVFF domain. (A) Model representing the mutation of the KVFF domain to KAFA on MR. (B) HEK-293 cells were transfected with GFP-MR<sup>wt</sup> or GFP-MR<sup>KAFA</sup> and treated with vehicle or aldosterone (10nM). The localization of the two proteins was analyzed by a fluorescence microscope. (C) HEK-293 cells were co-transfected with Stag-MR<sup>wt</sup> or Stag-MR<sup>KAFA</sup> and Flag- PP1α and treated or not with aldosterone (10 nM) for 3 h before lysis. Cell lysates were submitted to Pull-Down with Stag agarose beads and blotted with anti-Flag for Pull-down control. Expression level of proteins under investigation was determined by direct immunoblotting (Input) using Stag or Flag antibodies and actin as loading control. (D) HEK-293 cells were transfected with Stag-MR<sup>wt</sup> or Stag-MR<sup>KAFA</sup> with or without Flag- PP1α. Cell lysates were analyzed by immunoblotting using antibodies against Stag, Flag and actin as a loading control. Immunoblots shown are representative from 2 independent experiments.

**Figure 5:** PP1α stabilizes MR by inhibiting Mdm2. (A) mCCD<sub>cll</sub> cells were treated or not with Tautomycin (100nM for 16). Cell lysates were analyzed by immunoblotting using antibodies against phospho-Mdm2, Mdm2 and actin as a loading control. (B) HEK-293 cells were transfected with Flag-PP1α and cell lysates were analyzed by immunoblotting using antibodies against phospho-Mdm2, Mdm2, Flag and actin as a loading control. (C) PLA assay detecting endogenous MR-Mdm2 interactions in mCCD<sub>cll</sub> cells treated with vehicle or 100nM Tautomycin. After the PLA reaction, coverslips were mounted in the presence of DAPI to counterstain nuclei. (D) Quantitative analysis of PLA signals in mCCD<sub>cll</sub> cells from experience (C) in 3 independent stainings. Bars represent the means of discrete dots per cells ± SEM (n=80), \*\*\*P<0.001, Control vs. Tauto. (E) HEK-293 cells were co-transfected with Stag-MR, HA-Ub and Myc-Mdm2 and treated with MG132 (10μM) 6h before lysis. Cell lysates were submitted to Pull-Down (PD) with Stag agarose beads and the ubiquitylated forms of MR were analyzed by

immunoblotting with anti-HA antibody. Expression levels of MR and Mdm2 were determined by direct immunoblotting (Input) and actin as loading control. **(F)** HEK-293 cells were co-transfected with Stag-MR and increasing doses of Myc-Mdm2. The expression of proteins under investigation was determined by direct immunoblotting with anti-Stag or anti-Myc antibodies and actin as a loading control. **(G)** HEK-293 cells were co-transfected with Stag-MR and Myc-Mdm2 with or without Flag-PP1 $\alpha$ . Cell lysates were analyzed by immunoblotting using antibodies against Stag, Flag, Myc and actin as a loading control. **(H)** HEK-293 cells were transfected with Stag-MR and treated with Tautomycin (100nM) or Nutlin-3 (20 $\mu$ M) for 16h. Cell lysates were analyzed by immunoblotting using antibodies against Stag, Mdm2 and actin as a loading control. (A to H) Immunoblots shown are representative from 2-3 independent experiments.

**Figure 6:** PP1 $\alpha$  potentiates the aldosterone-induced transcription. **(A)** HEK-293 cells were co-transfected with Stag-MR with or without Flag-PP1 $\alpha$  and treated with vehicle or Tautomycin (100nM for 16h) or aldosterone (10nM for 3h). Cytoplasmic and nuclear fractions were analyzed by immunoblotting with anti-Stag. As controls, the fractions were reprobbed for  $\alpha$ -GAPDH (cytoplasmic) and lamin B (nuclear). Immunoblots shown are representative from 2 independent experiments. **(B and C)** HEK-293 cells were transfected with mouse mammary tumor virus (MMTV)-luc, CMV-Renilla, and MR together with PP1 $\alpha$ . After 24h of transfection, cells were treated with vehicle or 10nM aldosterone **(B)** or an increasing dose **(C)** for 16 h; then firefly luciferase (from MMTV-luc) was measured and normalized to Renilla. Data are representative of 2 independent experiments and given as means  $\pm$  SD of triplicates, \*\*\*P<0.001, MR vs. MR+PP1 $\alpha$ . **(D)** Same as **(B)** with co-transfection of Mdm2. **(E and F)** mCCD<sub>cl1</sub> cells were treated with or without Tautomycin (100nM for 16h), aldosterone (10nM for 4h). **(E)** The abundance of SGK1 mRNA was measured by qRT-PCR. Data are representative of 2 independent experiments in triplicates and given are means relative to GAPDH  $\pm$  SD (n=3). Cell lysates were also subjected to immunoblotting using antibodies against SGK1 and actin as a loading control (representative of 3 independent experiments).

**Figure 7:** PP1 $\alpha$  inhibition prevents aldosterone-induced Na<sup>+</sup> uptake. mCCD<sub>cl1</sub> cells grown on collagen-coated permeable filters. Cells were treated with aldosterone 10nM and Tautomycin for 16h. **(A)** The transepithelial equivalent short-circuit current was calculated according to Ohm's

law from the measured values of transepithelial potential difference and resistance (**B**). Data are representative of 3 independent experiments in triplicates. Each bar represents the mean  $\pm$  SD.



Table-1: Primer sequences used for qRT-PCR analysis

Primers	Sequences
MR fwd	AGTTCCTTTCCGCCTGTCAA
MR rev	TGACACCCAGAAGCCTCATC
PP1 $\alpha$ fwd	AAGAACGTGCAGCTGACAGA
PP1 $\alpha$ rev	GCCATGGATGTCACCACAGAT
Sgk1 fwd	CTCAGTCTCTTTTGGGCTCTTT
Sgk1 rev	TTTCTTCTTCAGGATGGCTTTC
Gapdh fwd	CCATCACCATCTTCCAGGAG
Gapdh rev	TCCATGGTGGTGAAGACAC
18sRNA fwd	GCAATTATTCCCCATGAACG
18sRNA rev	GGGACTTAATCAACGCAAGC
Podocalyxin fwd	ACA CAC AAA CCA TTG GGC AC
Podocalyxin rev	GTG TGG AGA CGG GCA ATG TA
Aminopeptidase N fwd	AATCTCATCCAGGGAGTGACC
Aminopeptidase N rev	GTGGCTGAGTTATCCGCTTT
NKCC2 fwd	GGA ATT GGT CTG GGC GTC A
NKCC2 rev	ATT GAC CCA CCG AAC TCA GG
NCC fwd	TGACCTGCATTCATTCCTCA
NCC rev	GAAGCGAACAGGTTCTCCAG
Aqp-2 fwd	CCGCCATCCTCCATGAGATT
Aqp-2 rev	TGCATTGTTGTGGAGAGCAT

Figure 1  
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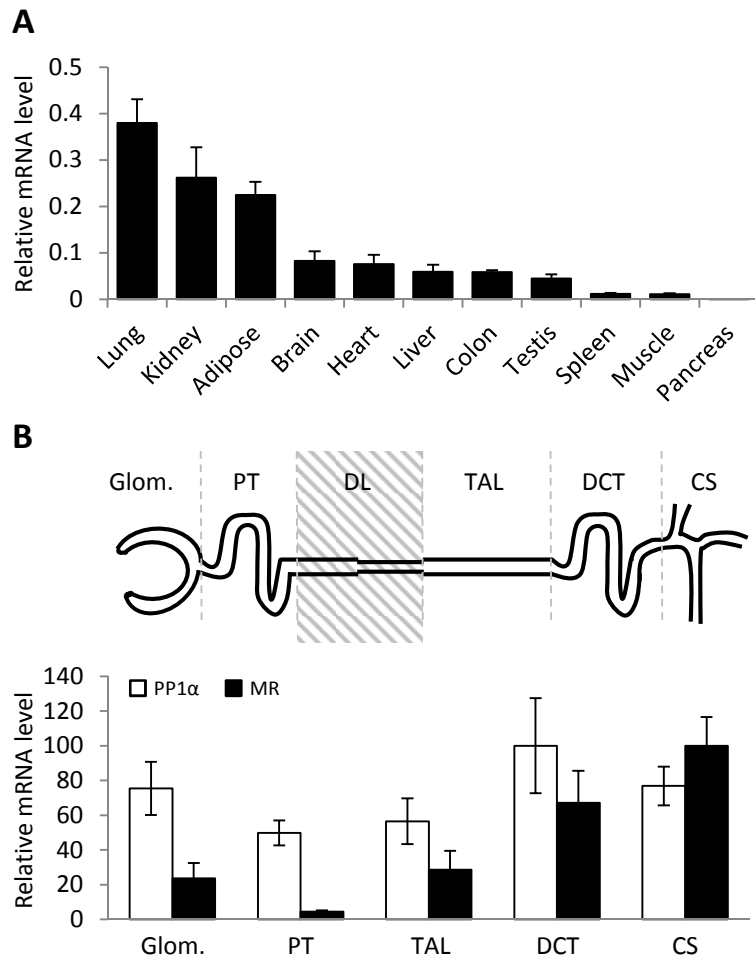


Figure 2  
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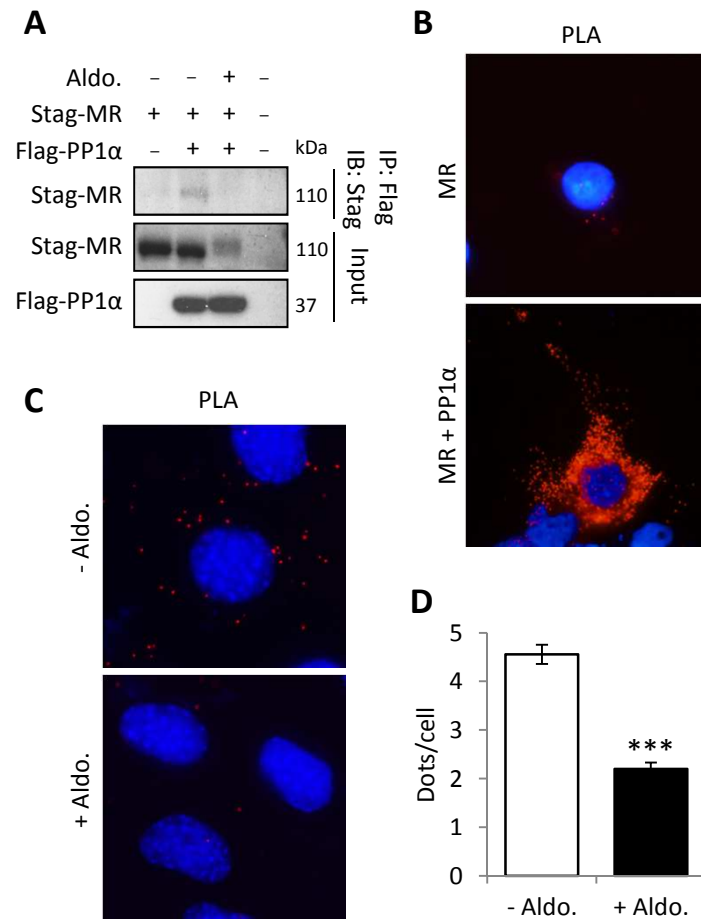


Figure 3  
Nagarajan S et al.

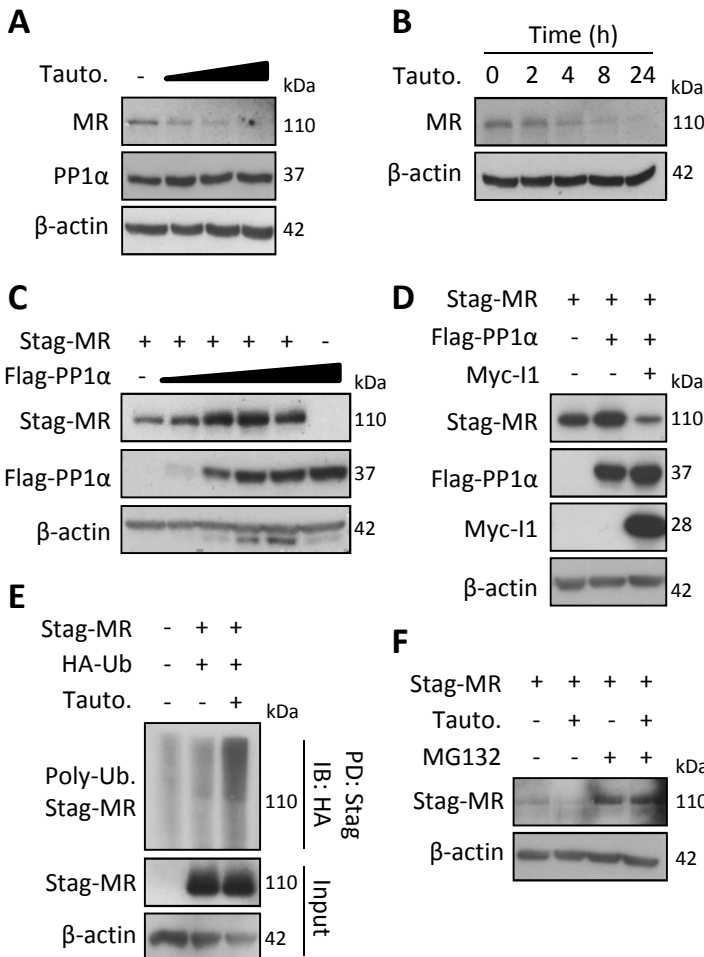


Figure 4  
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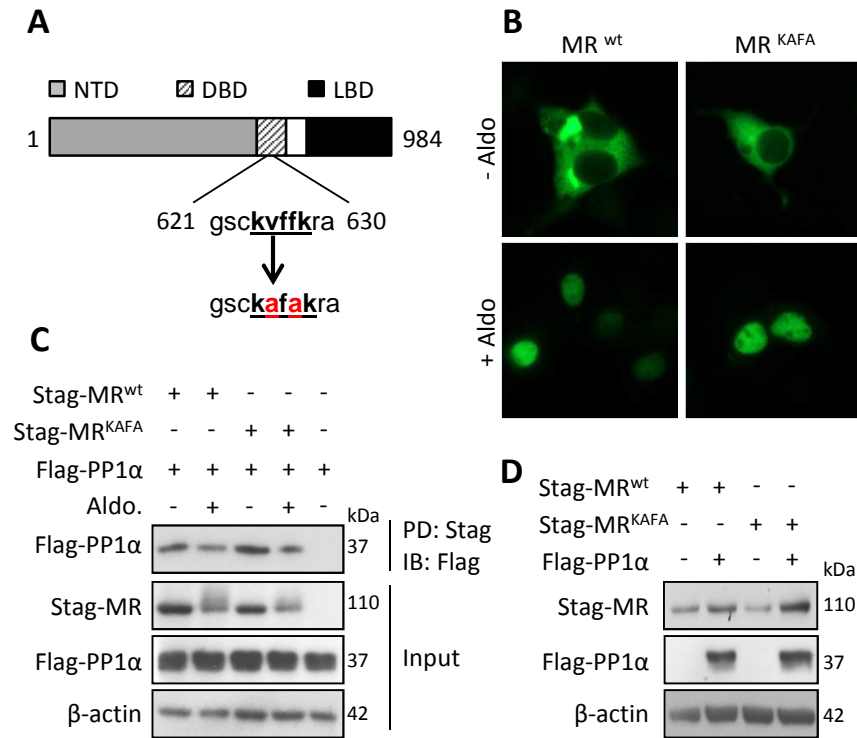


Figure 5  
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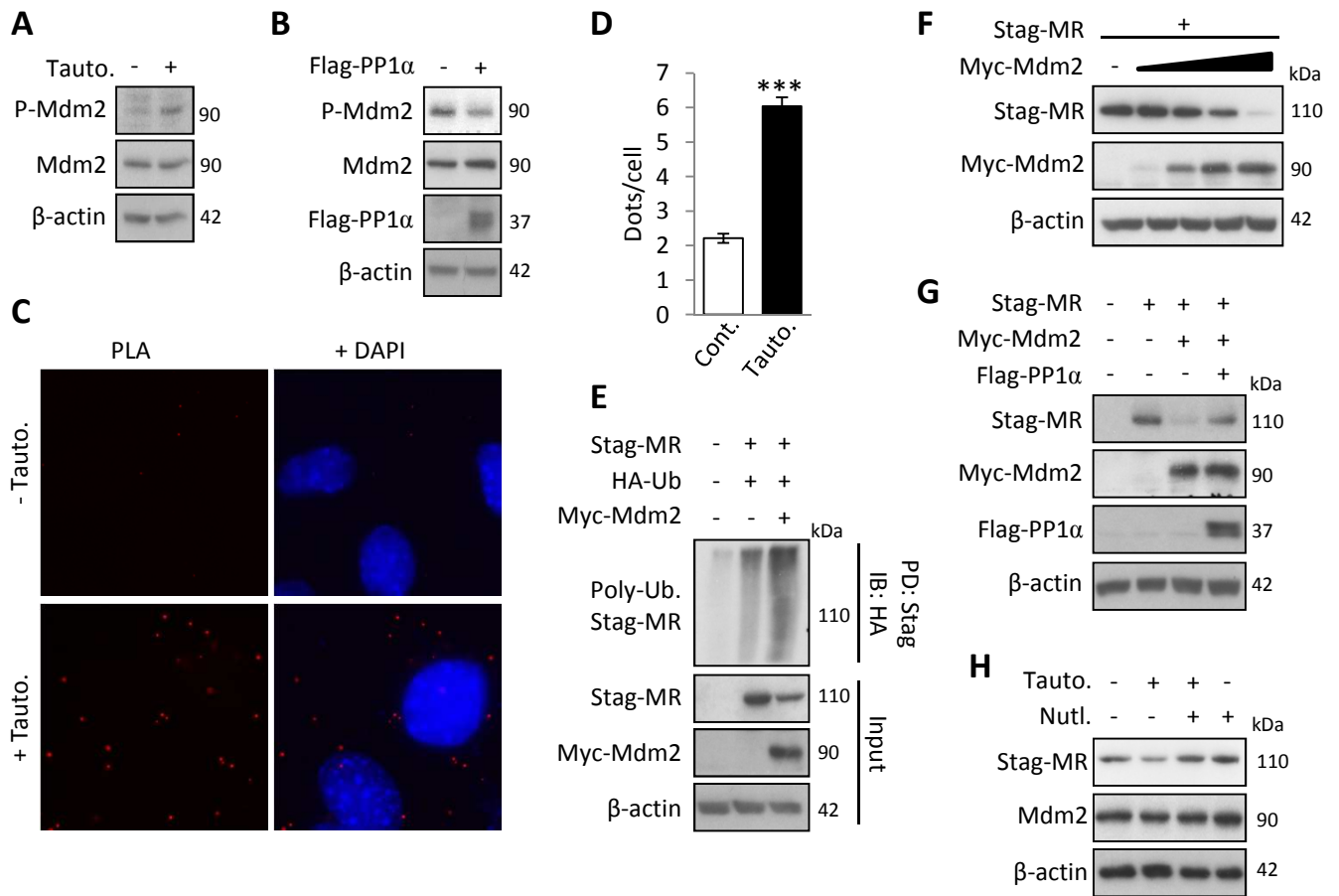


Figure 6  
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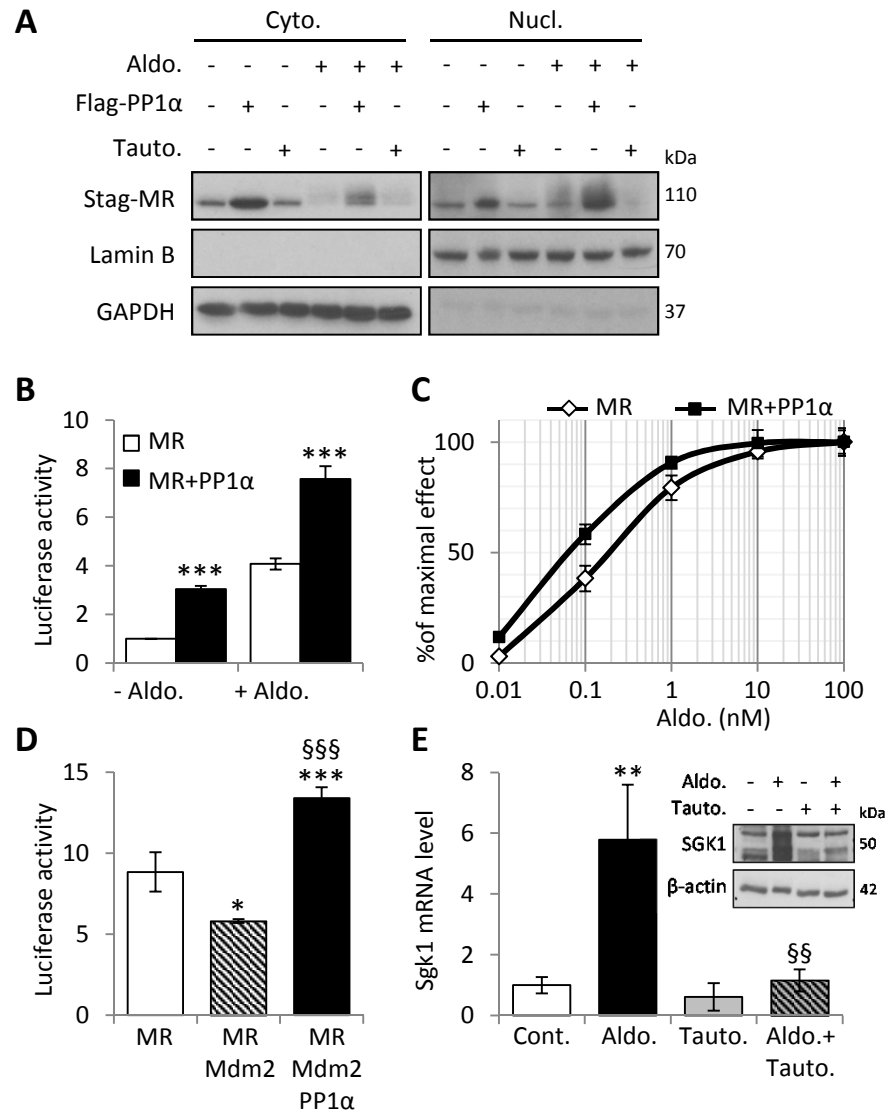


Figure 7  
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